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(54) Title: AN IMPROVED METHOD OF CLONING DOUBLE-STRANDED RNA, AND ITS USE IN THE PRODUCTION OF VIRAL GENE CLONES

(57) Abstract

A method of direct insertion of double-stranded RNA into a cloning vector consisting of double-stranded DNA, the method comprising: a) isolating the dsRNA from a source of interest; b) if required, decapping the dsRNA; and c) directly ligating the dsRna to the dsDNA of the appropriate cloning vector. This method allows dsRNA to be cloned directly, thus eliminating the traditional step of first making a complementary DNA copy of the RNA molecule. Using this method, it is possible to construct probes for the detection of virus diseases in plants and animals. The method can also be used to produce new plant strains which are resistant to a particular virus, or to provide immunity to animals to a specific virus.

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TITLE:

AN IMPROVED METHOD OF CLONING DOUBLE-STRANDED RNA, AND ITS USE IN THE PRODUCTION OF VIRAL GENE CLONES

TECHNICAL FIELD

The present invention relates to an improved method of cloning double-stranded RNA. In particular, it is directed to a method of cloning double-stranded RNA and the production of cloned viral genes which can be used to detect viral infection in plants and animals. The present invention can also be used to produce new plant strains which are resistant to a particular virus, or to provide immunity in animals to a specific virus.

BACKGROUND ART

Replication of viral RNA

The majority of known plant viruses contain single-stranded ribonucleic acid (ssRNA) as their genetic material. It is known that, during replication in their host plant, such viruses can produce not only double-stranded RNA (dsRNA) molecules corresponding to the complete ssRNA genome (replicative-form dsRNA, or RFs), but also dsRNA molecules smaller than RFs (replicative intermediates

[RIs]). These dsRNA molecules are present in relatively low amounts, but are very resistant to RNAse degradation and can therefore be readily isolated from infected plant material.

In many cases, there are more subgenomic sizes of dsRNA molecules (RIs) than the ssRNA which is packaged in the virus particles. These smaller dsRNAs may represent breakdown products formed during replication of the virus, but it is thought more likely that they represent single genes or functional units.

A few examples of plant viruses with ssRNA genomes and dsRNA RFs and RIs are tobacco mosaic virus (TMV), alfalfa mosaic virus (AMV), and luteoviruses such as barley yellow dwarf virus (BYDV) and beet western yellows virus (BWYV).

Another group of plant viruses have dsRNA genomes in the virus particles. With such viruses, there may be more than one size of RNA packaged. An example of such viruses is Fiji disease virus of sugar cane, which has about ten dsRNA molecules of different sizes in its particles. These molecules can be separated, as can the RF and RI molecules, electrophoretically according to size.

Certain viruses, with either ssRNA or dsRNA genomes, are only present at very low concentrations in specific tissues of infected plants. For example, luteoviruses such as BYDV and BWYV can only be found in phloem tissue and are therefore difficult to isolate in large quantities (approximately 1mg of virus can be isolated from 1kg of plant tissue, and of this virus only about 20% is RNA). During replication, these luteoviruses form dsRNA molecules, which can be isolated at concentrations of approximately 50µg/kg plant tissue.

The dsRNA Fiji disease virus (FDV) is similarly located mainly in particular areas of the sugar cane plant, and is therefore also difficult to isolate in large quantities.

For further study of such RNA viruses, it would be very useful to have cloned virus genomes and single genes, as these could then be rapidly multiplied in large quantities in microorganisms rather than whole plants. However, the low concentration of RNA means that cloning methods must be very efficient if full-length virus genomes are to be obtained.

Cloning of viral genes

In order to clone genes of RNA viruses in bacteria (which contain double-stranded deoxyribonucleic acid (dsDNA) as their genetic material) it has previously been necessary to first make a single-stranded complementary DNA (cDNA) copy of the viral RNA using the enzyme reverse transciptase. The cDNA copy is then usually converted to a double strand of DNA using DNA polymerase or reverse transcriptase. The double-stranded DNA copy can then be inserted into a bacterial DNA plasmid by standard recombinant DNA techniques, usually involving either blunt end ligation or ligation via synthetic DNA linkers or annealing after polynucleotide tailing of insert and plasmid DNA to give complementary ends.

Although this technique has been used to clone genes of a variety of RNA viruses, the production of double-stranded DNA copies is expensive and laborious. Moreover, even when the viral RNA is pure and plentiful, with long viral RNA genomes, the chance of obtaining full-length DNA clones is very low due to the reverse transcriptase and DNA polymerase enzymes "falling off" the RNA or cDNA strand.

Using standard techniques for converting RNA to complementary DNA (cDNA) copies for cloning, usually no more than approximately 0.1% of cDNA molecules obtained will be full-length, thereby dramatically reducing the frequency of obtaining complete cloned genomes for small amounts of starting RNA. Such techniques appear to be particularly inefficient for dsRNA viruses, probably because the dsRNA molecules are very tightly bound together, preventing easy access for the reverse transciptase or DNA polymerase used to synthesize cDNA copies. To date, however, cDNA techniques have been the only method used to clone RNA genomes of both plant and animal viruses.

If dsRNA molecules could be cloned by some method other than via inefficient cDNA procedures (the only currently available method), this would facilitate cloning of full length, functional genes of viruses with ss or dsRNA genomes, and would be a particularly valuable technique for those viruses present at low concentrations in infected plants.

Detection of virus infections

There are several methods available for detection of virus diseases in plants. These include a study of the symptoms caused in test plants, microscopic examination of virus particles from infected tissue, ELISA immunological assays, and probing of infected

material with virus-related nucleic acid. Of these methods, the last is the most specific, rapid, and simple test available, with the added advantage that many different samples can be tested at the same time, with quantitative results obtained in a few days or less.

Two types of probes can be used: labelled cDNA made from isolated viral RNA, or a labelled probe made from cloned viral genes. If cDNA is used, then it is necessary to have a supply of viral RNA for this purpose. Isolation of viral RNA, even when it is present in plants in high concentrations, is a laborious and time-consuming procedure. Moreover, when virus levels are low in infected material, the isolation of RNA is even more difficult.

Cloned viral genes, however, overcome this problem. Microorganisms containing the viral inserts can be easily grown, and vectors with the inserts can be isolated and purified in large quantities by simple, rapid procedures. Virus-related inserts can then be used to manufacture probes either by nick-translation or by cDNA synthesis after DNA strand separation.

Thus cloned viral gene sequences are the method of choice for probe construction.

Of commercial significance, is the production of probes for virus detection in plants.

Similarly, cloned viral genes can be used to construct probes for the detection of animal viruses and for virus-like diseases of plants and animals.

Control of virus infections

The use of viral genes for the biological control of viruses is an area of great interest at present, and attempts have been made to control viruses of plants by transferring viral genes for cross-protection into them.

Cross-protection, also known as super-infection immunity or viral interference, is the specific resistance shown by a virus-infected host cell when attempts are made to infect it a second time with the same virus or with a closely related one.

The cross-protection produced by avirulent virus variants is already used as a virus control measure in certain crops. For example, it is used to control passion fruit woodiness potyvirus in passion fruit orchards, and to control citrus tristeza closterovirus in citrus crops. It was, until recently, also widely used to control tomato mosaic tobamovirus in tomato crops.

Cross-protection has not been more widely used as a control measure as there has been concern that a deliberately spread avirulent virus, though protecting its chosen host, may be virulent in other species and spread to them, causing even greater problems than the original one.

However, cross-protection would be particularly valuable against the luteoviruses. Luteoviruses are transmitted by aphids and, although they do not replicate in the aphids, they are carried for life. Luteoviruses have isometric particles about 25nm in diameter; each contains a genome, which consists of a single molecule of single-stranded RNA with a molecular weight of about 2 million. They replicate only in plant phloem cells and hence the virions attain very small total concentrations in plants. They disrupt translocation in infected plants, causing yellowing, reddening and leaf rolling.

Luteoviruses can be effectively controlled by treating crops with insecticides or by destroying virus and aphid sources. However, although these methods are suitable for controlling luteoviruses of intensively-grown high-value crops such as sugar beet or potatoes, they are not economical for large acreage low-value crops such as cereals or pastures. For these it would be best to incorporate genetic resistance in the crop species. Naturally occurring

resistance genes have been found and moved into desirable cultivars of some crop species using standard plant breeding methods. The process is very time-consuming, and it has often proved difficult to conserve other desirable characters (e.g. resistance to other pathogens). Resistance of this sort has been bred into sugar beet for controlling luteovirus losses and barley yellow dwarf virus (BYDV)-resistance has been successfully incorporated into a commercial barley variety. Unfortunately suitable resistance genes to most luteoviruses have not been found.

The isolation of the viral genes for cross-protection, and transfer of these genes into the genome of the host so that they are inherited and expressed, will overcome the problem of spreading of whole viruses to other hosts.

dsRNA cloning methods can be used to obtain single functional virus genes. These can then be linked, using standard recombinant DNA techniques, to a promoter which functions in plant cells, thereby enabling the viral gene product to be manufactured in plant cells.

The linked promoter viral gene construct can then be transferred into a modified tumour-inducing (Ti) plasmid of Agrobacterium, in such a way that, on

infection of appropriate plant cells, the viral gene construct is incorporated into the plant genome together with parts of the Ti plasmid vector.

After culture of the plant callus containing the viral insert, regeneration of plantlets can be initiated by appropriate hormone treatment. Plants thus obtained will have the viral gene present in cells throughout the whole plant, and these can be tested for resistance to infection by the same or related viruses. Plants with the cross-protection gene(s) inserted will resist superinfection with such viruses.

Thus new plant varieties can be constructed containing virus cross-protection genes, which are not normally present in the plant genomes.

The phenomenon of cross-protection is also known to operate among viruses of animals. Therefore, the molecular introduction of cross-protecting viral genes is applicable to animals as well as plants.

Production of viral RNA, proteins and antisera

Cloned viral dsRNA can be used to obtain large quantities of very pure RNA and protein encoded by the cloned gene, by in vitro transcription and translation of the DNA insert. The RNA thus obtained

can be used for infectivity tests on plant material, either alone or in combination with other viral RNAs. The protein can be used for in vitro packaging tests of viral genetic material, or for production of specific antisera. This is of importance in the production of specific antisera made from pure viral proteins, particularly proteins not normally isolated in large quantities from infected cells.

Virus gene sequencing

In order to determine the number and types of genes present for each virus, cloned genetic material is essential for sequencing of the viral genomes. For this purpose, the dsRNA of RFs and RIs is most useful for cloning single, functional units for gene sequencing. Where levels of viral RNA are low, and where the whole viral genome has not been sequenced, clones obtained from dsRNA should facilitate identification and sequencing of these viral genes. Sequencing is a research tool for understanding the makeup of the genome and the way in which it functions.

DISCLOSURE OF THE INVENTION

It is an object of the present invention to overcome the problems associated with current cloning procedures and to directly clone dsRNA, in which the traditional step of first making a cDNA copy of the RNA molecule is eliminated.

By direct cloning, it is meant that viral dsRNA is itself ligated to the dsDNA of cloning vectors to be subsequently used for transformation in recipient cells such as Escherichia coli, rather than first making a cDNA copy of the RNA molecule. During replication of the DNA vector containing the dsRNA insert, the RNA portion is converted to dsDNA by the replicative enzymes of the host cells.

A second object is the use of this direct cloning of dsRNA to construct probes for the detection of virus diseases.

A third object is the use of direct dsRNA cloning to obtain functional viral genes which can be transferred to plants to confer specific resistance to particular viruses, free from the potential problems that come from the use of avirulent virus variants.

The present invention allows the laborious step of producing double-stranded DNA copies in vitro to be eliminated by allowing double-stranded RNA to be cloned instead of DNA.

According to a first aspect of the present invention, there is provided a method of direct insertion of the double-stranded RNA into a cloning vector consisting of double-stranded DNA, said method comprising:

a) isolating the dsRNA from a source of interest;
b) if required, decapping the dsRNA; and
c) directly ligating the dsRNA to the dsDNA of the appropriate cloning vector.

It has been discovered that a number of techniques can be employed in the direct ligation of the dsRNA to the dsDNA cloning vector.

It has been established that conventional synthetic dsDNA linkers, after ligation to the dsRNA, enable the thus-modified dsRNA to be directly ligated to plasmid dsDNA. Bam HI and Eco RI linkers have been found to be particularly suitable.

Alternatively, the dsRNA molecules can be first tailed, for example, with poly-A or poly-C, and then ligated to plasmid dsDNA which, in turn, has also been tailed with, for example, poly-dT or poly-dG.

Using this procedure, homopolymer DNA tails must be added to the ends of the plasmid DNA which are complementary to the homopolymer RNA tails added to the dsRNA. The tailed dsRNA and plasmid dsDNA are then subjected to annealing by conventional techniques.

The dsRNA can also be directly ligated to dsDNA by first ensuring that all ends of the dsRNA are completely blunt. The blunt-ended dsRNA is then ligated to a suitable blunt-ended DNA plasmid, once again, using standard techniques well-known in the art for blunt-end ligation.

Once the plasmid with dsRNA inserts has been constructed, it can be transformed into recipient cells such as Escherichia coli by standard techniques well known in the art. The transforments thus-produced can be isolated by methods also well known in the art, but preferably, by Southern hydridization or antibiotic sensitivity.

Direct cloning of dsRNA is most useful in two main situations: where very low amounts of RNA are available, or where single, functional genes are required.

The method of the present invention can be used to obtain cloned viral genes for a variety of purposes such as:

i) nucleic acid probes for viral infections, particularly where low levels of virus are present in the infected tissue;

ii) functional genes isolated from the whole viral genome for insertion into host plant genomes for viral cross-protection;

iii) production of large amounts of viral RNA, by in vitro transcription;

iv) viral gene sequencing;

v) in vitro protein production from cloned viral genes via in vitro transcription and translation.

The dsRNA cloning method of the present invention is a simple method for obtaining inserts suitable for use as probes.

For example, clones of AMV, TMV and FDV obtained from dsRNA have been used to probe plant material which was either known or suspected to be infected with the appropriate virus.

It should be noted that these methods, while described for particular plant viruses, could also be applied equally well to a whole range of other plant and animal viruses.

It would also be possible to use the dsRNA cloning method with ssRNA molecules, if these are first converted to dsRNA (rather than cDNA) with an enzyme such as RNA-dependent RNA polymerase or reverse transcriptase.

Using the present invention, new plant varieties, similar to varieties currently available commercially, except for the incorporation of part of a virus genome encoding cross-protection, could be produced. Virus infection can cause a crop loss in, for example, alfalfa of 2-5% which should be overcome if the alfalfa mosaic virus cross-protection gene is inserted.

In addition to cross-protection against alfalfa mosaic virus in alfalfa, the present invention is applicable to cross-protection against tobacco mosaic virus in tobacco and tomatoes, beet western yellows virus in sugarbeet and soybeans and barley yellow dwarf virus in barley, wheat, rice and oats. However the technique could be equally well applied to a wide range of other viruses which infect plants or animals.

Details of the materials and methods as used in the present invention will now be described. In this description, all temperatures are in degress centigrade, and all technical terms and abbreviations

have the usual meanings in the art. Products can be isolated and purified by the means described herein, or by other means known in the art.

DETAILED DESCRIPTION OF EMBODIMENTS

Isolation of virus-related dsRNAs (RFs and RIs)

Virus-infected plants were homogenized in an appropriate buffer (eg. 50mM Tris, 100mM NaCl, 1mM EDTA, pH 7.0) containing a small amount of mild detergent (eg. 2% w/v SDS) and an antioxidizing agent (eg. 1% v/v 2-mercaptoethanol). The slurry was further homogenized with phenol, and then clarified with chloroform. After a low-speed centrifugation, ethanol was added to the aqueous phase to 15% v/v final concentration. Under these conditions, dsRNA is preferentially bound to a Whatman CF11 cellulose column and can be subsequently eluted in the absence of ethanol. Routinely, two CF11 columns were used to bind and elute the viral dsRNA.

The eluted nucleic acids were treated with DNAse to remove any traces of DNA. The dsRNA was then phenol-chloroform extracted and precipitated with 0.3M sodium acetate and ethanol to purify and concentrate it.

In some experiments, the dsRNA molecules were fractionated by electrophoresis in polyacrylamide or agarose gels and distinct sizes were eluted from the gels.

Isolation of FDV RNA

Fiji disease virus dsRNA was extracted directly from galls of infected sugarcane following a procedure similar to that described above for RFs and RIs.

Decapping of RNA

dsRNA was treated with tobacco acid phosphatase (TAP) from Bethesda Research Laboratories to remove any possible "cap" structures from the ends of the molecules. The presence of such structures might inhibit ligation of these molecules to dsDNA.

The decapping reaction was carried out as described by Bethesda Research Laboratories in 50mM sodium acetate buffer pH5.0 in the presence of 10mM 2-mercaptoethanol and 1mM EDTA, for 60 minutes at 37°C. After extraction with phenol and ethanol precipitation, the dsRNA molecules were treated with polynucleotide kinase to restore phosphate groups to the 5'-OH termini prior to ligation.

In general, the decapping procedure was not found to affect ligation of dsRNA to DNA molecules.

Cloning methods for dsRNA

Unless otherwise stated, enzymes, buffers and methods were as described in Molecular Cloning by T. Maniatis, E.F. Fritsch and J. Sambrook, Cold Spring Harbor (1982). Restriction enzymes, synthetic linkers, T4 DNA ligase and polynucleotide kinase were purchased from New England Biolabs; reverse transcriptase from Life Sciences Inc; DNA polymerase (Kornberg and Klenow) from Boehringer Mannheim; T4 RNA ligase from PL Biochemicals; and terminal transferase from New England Nuclear.

i) Addition of linkers

Synthetic dsDNA linkers were first phosphorylated using T4 polynucleotide kinase. These were then ligated to the dsRNA (blunt end ligation) using T4 DNA ligase and T4 RNA ligase. High concentrations of linkers (up to 0.2 ug/10 ul) and both ligases (up to 8U RNA ligase and 200U DNA ligase/10ul) were used to ensure that ligation proceeded as far as possible. In these experiments, Bam HI and

Eco RI linkers were used but a variety of other synthetic molecules could be used in a similar manner.

When ligation had occurred, the ligases were inactivated at 65°C and the linkers were cut with the appropriate restriction enzyme (in this example Bam HI or Eco RI). Under standard conditions for restriction enzymes, these enzymes were not observed to cut dsRNA molecules. Therefore only the DNA linkers, not the dsRNA, should be cut when a sequence is recognised by the restriction enzyme.

ii) Ligation to plasmid DNA

Plasmid DNA (eg. pBR322, pBR325) was isolated, from E. coli strain RR1, purified on Cs Cl gradients, and cut with the appropriate restriction enzyme. The linear DNA was purified by elution of the band formed on an agarose gel. It was then usually treated with calf intestine alkaline phosphatase to remove termal 5' phosphates, to prevent self-ligation and thereby minimize the background of non-recombinant plasmids.

The dsRNA molecules with attached linkers, after cutting with restriction enzymes, were ligated to appropriate linearized plasmid DNA with T4 DNA ligase.

iii) Poly-A and poly-C tailing by dsRNA

dsRNA molecules were tailed with poly-A or poly-C, the ss tails being 20-100 nucleotides long. Poly(A) polymerase was used to add AMP (derived from ATP) or CMP (from CTP) on to the free 3'-OH termini of the dsRNA molecule. The reaction was carried out in a buffer containing 50mM Tris pH 7.6, 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.2 mM rATP, 0.2M NaCl and 2.5mM $MnCl_2$ at 37^{o} for 15 min. The number of AMP or CMP molecules added to the 3' ends of the RNA was monitored by using ^{3}H-rATP or ^{3}H-rCTP.

iv) Poly-dT and poly-dG tailing of plasmid DNA

PstI-cut, linear plasmid molecules were tailed with dT or dG residues, the tails being 20-100 nucleotides in length. Terminal transferase was used to catalyze the addition of deoxynucleotides to the 3'-OH termini of the DNA molecule. The number of deoxynucleotides added to the 3' ends of the plasmid DNA was

monitored by using ^{32}P-dATP and ^{32}P-dCTP, and then similar conditions were used with unlabelled dTTP and dGTP to obtain 20-100 nucleotide tails. With this treatment, homopolymer DNA tails were added to the ends of the plasmid molecule which were complementary to the homopolymer RNA tails added to the dsRNA.

v) Annealing of tailed RNA and DNA

Approximately equimolar amounts of dsRNA and plasmid DNA, tailed with complementary tails, were heated to 65°C for 5 min, incubated at 57°C for 2h and then cooled slowly to room temperature in annealing buffer. The RNA and DNA were present at final concentrations of approximately 1ug/ml in the annealing mixture.

vi) Blunt-end ligation

dsRNA was treated in DNA repair buffer with 10U/20ul DNA polymerase (Klenow fragment) in the presence of all four 0.1M dNTPs to ensure that the ends were completely blunt. Alternatively, the dsRNA was treated with S1 nuclease (500-1000 units at room temperature for 30 min) to remove any ssRNA ends. However, these treatments were not found to be

necessary for AMV and TMV RFs and RIs and FDV dsRNA, presumably because these molecules are already blunt-ended.

A plasmid such as pUC8 was cut with a restriction enzyme such as SmaI, to give a blunt-ended linear molecule. This was then ligated to the dsRNA using high concentrations of T4 DNA ligase and T4 RNA ligase as described above for ligation of synthetic linkers.

The constructed plasmids were transformed into Escherichia coli strain RR1. Clones with viral gene inserts were isolated from the colonies obtained by Southern hybridization with a radioactive cDNA probe made from viral RNA with reverse transcriptase and random primers.

Once inside the E. coli cells, the dsRNA inserts are converted to dsDNA during plasmid replication. After plasmid reisolation, the inserts can then be cut with restriction enzymes and treated in the same way as clones obtained by traditional cDNA methods.

AMV dsRNA

AMV-related dsRNA molecules were isolated from Nicotiana tabacum var. Samsun and from N. tabacum var. White Burley. Up to 7 different sized dsRNA molecules were detected on 10% Laemmli acrylamide gels, with a size range of approximately 0.5 kilobases (kb) to 4 kb. The relative concentration of dsRNA molecules of different size varied depending on the variety of N. tabacum used, and the time of isolation after infection of plants with AMV.

AMV dsRNA molecules have been cloned by the addition of synthetic DNA linkers, or by tailing of molecules. In the first case, Bam HI dsRNA linkers were ligated directly to the dsRNA. The ligated molecules were digested with the restriction enzyme Bam HI, which only recognized Bam HI DNA sequences and not RNA sequences, thereby avoiding the digestion of AMV genes which contain Bam HI recognition sites. (For example, if standard cDNA techniques had been used, Bam HI would have cut the AMV3 cDNA copy, but this did not occur with the dsRNA gene, which is another advantage of cloning dsRNA directly).

After insertion of the dsRNA moleclules with Bam HI dsDNA linkers into plasmid pBR322 cut with Bam HI using T4 DNA ligase, the mixture was transformed into Escherichia coli strain RR1. Recombinant clones containing AMV genes were isolated by Southern

hybridization with a total AMV cDNA radioactive probe. Positive clones were then purified, and their AMV inserts further characterised.

AMV dsRNA molecules were also cloned by polynucleotide tailing. Single-stranded poly-A or poly-C tails were added to the 3' ends of the RNA using poly(A) polymerase from E. coli. This enzyme is normally used to add poly-A tails to 3' ends of ssRNA molecules prior to cDNA synthesis primed with a complementary poly-dT primer. However, it has been found that the same enzyme preparation can add poly-C tails, albeit less efficiently than poly-A, to the 3' ends of dsRNA molecules.

AMV dsRNAs with poly-A tails (approximately 20-100 added nucleotides) were annealed with plasmid pBR322 cut with restriction enzyme Pst I and treated with terminal transferase to add poly-dT tails approximately 20-100 nucleotides in length. Similarly, AMV dsRNAs with poly-C tails were annealed with Pst I cut pBR322 with poly-dG tails. The latter reaction has the advantage that a Pst I site can be recreated at each end of the AMV insert, allowing later removal of the insert from the plasmid DNA for further study.

After transformation of the annealed plasmid mixtures into E. coli strain RR1, clones with AMV inserts were identified by Southern hybridization with an AMV cDNA probe.

Many different AMV dsRNA clones have been obtained by these methods. The most efficient method of cloning the dsRNA is by addition of poly-A tails.

TMV dsRNA

TMV dsRNA molecules have been isolated from N. tabacum var. Samsun and separated on 10% Laemmli gels. These dsRNAs, like those of AMV, have been cloned after addition of poly-A tails using E. coli poly(A) polymerase.

A variety of TMV dsRNA clones have been obtained by this method.

FDV

Fiji disease virus genomic dsRNA was isolated directly from infected sugarcane. The dsRNA was separated on a 10% Laemmli acrylamide gel, and RNA sizes ranged from approximately 3.5 kb to 9 kb.

The dsRNA could be directly cloned by the addition of DNA linkers, by tailing of molecules, or by direct blunt-end ligation into a plasmid.

FDV dsRNA has been cloned by addition of Bam HI linkers or Eco RI linkers, followed by digestion with the appropriate restriction enzyme and ligation into Bam HI-cut pBR322 or Eco RI-cut pBR325 respectively. Selection of clones with FDV inserts was by Southern hybridization with a FDV cDNA probe. The clones with FDV sequences were then purified and further characterized.

FDV dsRNA has also been cloned directly after addition of poly-A tails by annealing with Pst I-cut pBR322 tailed with poly-dT, as described above for AMV dsRNA. Different FDV clones were identified by Southern hybridization with a FDV cDNA probe.

DISCUSSION

Using the present invention, it is now possible to directly clone dsRNA, in which the traditional step of first making a complementary DNA copy of the RNA molecule is eliminated.

Whichever technique of achieving insertion of dsRNA into plasmids is used, it is the bacterial cell rather than *in vitro* techniques which converts

double-stranded RNA into a double-stranded DNA copy during normal replication of the plasmid which accompanies cell division.

Using this technique, it is possible to obtain clones for sub-genomic fragments of double-stranded viral RNA as well as full-size genomic clones.

The technique can be applied equally well to direct cloning of viral genomes which consist of double-stranded rather than single-stranded RNA. In these cases, the RNA can be isolated either from virus particles or directly from infected tissue.

Similarly, double-stranded RNA from any source can be used to clone genes directly rather than using DNA copies.

A number of different clones of plant virus genes have been obtained by the method of the present invention as described above. In particular, single genes of alfalfa mosaic virus have been cloned from double-stranded RNA by addition of Bam HI linkers and insertion into Bam HI-cut pBR322 plasmid, and also by polynucleotide tailing and insertion in the Pst I site of pBR322.

Similarly, probes for TMV, FDV, BYDV and BWYV can be readily produced by the method of the present invention.

Those skilled in the art will appreciate that modification and variations to the invention described above are possible without departing from the present inventive concept.

CLAIMS

1. A method of direct insertion of double-stranded RNA into a cloning vector consisting of double-stranded DNA, said method comprising:

   a) isolating the dsRNA from a source of interest;
   b) if required, decapping the dsRNA;
   c) directly ligating the dsRNA to the dsDNA of the appropriate cloning vector.

2. A method as defined in claim 1, wherein the ligating of dsRNA to the dsDNA of the cloning vector is by blunt end ligation.

3. A method as defined in claim 1, wherein the ligating of dsRNA to the dsDNA of the cloning vector is via the addition of DNA linkers to the dsRNA molecule.

4. A method as defined in claim 1, wherein the ligating of dsRNA to the dsDNA of the cloning vector is by annealing after complementary polynucleotide tailing of the dsRNA and dsDNA.

5. A method of direct-insertion of double-stranded RNA into a cloning vector consisting of double-stranded DNA, said method comprising:

a) converting single-stranded RNA to double-stranded RNA;
b) isolating the dsRNA;
c) if required, decapping the dsRNA; and
d) directly ligating the dsRNA to the dsDNA of the appropriate cloning vector.

6. A method as defined in any one of claims 1 to 5, wherein the thus-ligated dsRNA is converted to dsDNA in a host cell containing the cloning vector with the thus-ligated dsRNA.

7. dsDNA molecules whenever produced from dsRNA by a method as defined in any one of claims 1 to 6.

8. Virus-related dsDNA molecules whenever produced from dsRNA by a method as defined in any one of claims 1 to 6.

9. Alfalfa mosaic virus dsDNA molecules whenever produced by a method as defined in any one of claims 1 to 6.

10. Tobacco mosaic virus dsDNA molecules whenever produced by a method as defined in any one of claims 1 to 6.

11. Fiji disease virus dsDNA molecules whenever produced by a method as defined in any one of claims 1 to 6.

12. Barley yellow dwarf virus dsDNA molecules whenever produced by a method as defined in any one of claims 1 to 6.

13. Beet western yellows virus dsDNA molecules whenever produced by a method as defined in any one of claims 1 to 6.

14. Material which has had inserted therein dsDNA molecules as defined in claim 7.

15. Plants which have been grown from material which has had inserted therein dsDNA molecules as defined in claim 7.

16. Plants, resistant to a specific virus, which have been grown from material which has had inserted therein dsDNA molecules isolated from the specific virus, said dsDNA molecules having been produced by a method as defined in any one of claims 1 to 6.

17. Alfalfa grown from material which has had inserted therein alfalfa mosaic virus dsDNA molecules as defined in claim 9.

18. Tobacco grown from material which has had inserted therein tobacco mosaic virus dsDNA molecules as defined in claim 10.

19. Sugar cane grown from material which has had inserted therein Fiji disease virus dsDNA molecules as defined in claim 11.

20. Barley, wheat, oats or rice grown from material which has had inserted therein barley yellow dwarf virus dsDNA molecules as defined in claim 12.

21. Sugarbeet or soybeans grown from material which has had inserted therein beet western yellows virus dsDNA molecules as defined in claim 13.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 85/00093

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C12N 15/00, C07H 21/04, A01H 1/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System [8]	Classification Symbols
IPC	C12N 15/00
US	435 - 172

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [9]

BIOSIS PREVIEWS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	METHODS IN ENZYMOLOGY, VOLUME 65, issued 1980. By DUNN, A.R. et al "Mapping Viral mRNAS by Sandwich Hybridization", pages 468-478	1-6
A	PROGRESS IN NUCLEIC ACID RESEARCH AND MOLECULAR BIOLOGY, VOLUME 11, issued 1971. By KENNEL; D.E., "Principles and Practices of Nucleic Acid Hybridization" pages 259-301.	1-6
A	US, A, 4,415,553 (ZHABILOV, H.P. et al) 15 November 1983 (15.11.83)	1-6
A	MOLECULAR AND GENERAL GENETICS, VOLUME 189, NUMBER1, issued 1983. By JARSCH, M et al, "Physical Organization of the genes for ribosomal RNA in Methanococcus vannielli", pages 41-47.	1-6
A	THE EMBO JOURNAL, Volume 3, Number 6, issued 1984. By KEMPERS-VEENSTRA, A, et al "Transcription of an Artificial ribosomal RNA gene in yeast", pages 1377-1382.	1-6

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1.8.85 (1 August 1985)	(07.08.85) 7 AUGUST 1985
International Searching Authority: AUSTRALIAN PATENT OFFICE	Signature of Authorized Officer: J.W. ASHMAN

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 15-21 because they relate to subject matter not required to be searched by this Authority, namely:

Plant varieties.

2. [ ] Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.